Chemoecology 16:9–16 (2006)
0937–7409/06/010009–8

DOI 10.1007/s00049-005-0320-x

CHEMOECOLOGY

Adaptation of a four-arm olfactometer for behavioural bioassays of large beetles

Imen Saïd[1], Rosa Aldana de la Torre[2], Jean-Paul Morin[3] and Didier Rochat[4]

[1]CNRS UMR 6552, Université de Rennes 1, Campus de Beaulieu, 35042 Rennes cedex, France
[2]Cenipalma, Calle 21 No. 42C-47, Santa Fé de Bogotá, Colombia
[3]UR 31, TA 80/02, Avenue de la Lironde, 34398 Montpellier cedex 5, France
[4]UMR 1272, Physiologie de l'Insecte: Signalisation et Communication, INRA, Route de Saint-Cyr, 78026 Versailles cedex, France

Summary. We adapted a four-arm olfactometer (55 × 55 × 5.5 cm) for bioassays of large insects and its usefulness was evaluated by testing the responses of three beetles, the palm weevil *Rhynchophorus palmarum* (L.) (Coleoptera: Curculionidea) and two Dynast scarabs *Strategus aloeus* (L.) and *Oryctes rhinoceros* (L.) (Coleoptera: Scarabaeidae) to plant odours and aggregation pheromones. This olfactometer was coupled to a volatile delivery system dispensing highly volatile semiochemicals at constant concentrations. We materialized airflows in the olfactometer by discolouring sensitive papers with acid vapours under various conditions (air straighten by stainless steel grids in inputs and output, 500 ml/min per arm) to visualize air turbulence and to test air-tightness. The volatile delivery system can be used to mix and dispense a broad range of concentrations of semiochemicals in air from diluted aqueous solutions. It was calibrated by measuring the release of the pheromone using solid phase-microextraction (SPME). Dose-response curves to synthetic pheromone were obtained for *O. rhinoceros* of both sexes. Coupling the volatile delivery system to the four-arm olfactometer provided a system with which the responses of *R. palmarum* and *S. aloeus* to their synthetic aggregation pheromones and to natural plant volatiles could be readily observed and studied.

Key words. Four-arm olfactometer – volatile delivery system – aggregation pheromone – palm beetles – Coleoptera – Curculionidae – Scarabaeidae – *Rhynchophorus palmarum* – *Oryctes rhinoceros* – *Strategus aloeus*

Introduction

Many large tropical insects, such as palm beetles, are major pests (*e.g.* Bedford 1980). As efficient control methods are lacking, chemical ecology studies of some species aim to develop olfactory trapping, particularly using pheromones. Chemical communication appears highly developed in palm beetles as in most Coleoptera. Various attractive pheromones and plant odours have been identified and evaluated in the field (Giblin-Davis *et al.* 1996; Hardie & Minks 1999; Rochat *et al.* 2000, 2002, 2004). Successful mass trapping using synthetic pheromones and plant co-attractants has been reported for *Rhynchophorus* spp., *Oryctes* spp. and *Scapanes australis* (Abraham *et al.* 2000; Chung 1997; Oehlschlager *et al.* 1992, 1995, 2002). For most of these species, the need to add plant material to synergize pheromones has limited implementation of olfactory trapping on a large scale. Often, mass trapping did not reduce damage in spite of important captures of pests. For example, this technique could not control *Rhynchophorus ferrugineus* invasions in the Middle East (Ollivier and Rochat, 2003).

These limits and failures could be due in part to lack of data concerning variability of olfactive responses of palm beetles and factors that could influence responses: age, sex, density, climatic conditions … None of these species have been the subject of detailed laboratory experiments measuring the effect of these factors on their behavioural responses to semiochemicals active in mass trapping.

So far, two constraining factors have limited the study of the behaviour of palm beetles in the laboratory: first, the very large size of these insects compared to most insect pests studied previously. And second, the difficulty to deliver the highly volatile semiochemicals they respond to (typically C2 to C10 molecules, *e.g.* ethanol, ethyl acetate, and 2-butanone: pheromone component of *Strategus aloeus*; Rochat *et al.* 2000a, b), at concentrations maintained constant over time using simple classical dispensers such as rubber septa, filter papers or glass capillaries (Baker and Cardé 1984).

We developed a tool to study, under laboratory conditions, behavioural responses to highly volatile semiochemicals, for example, the synergistic responses of palm weevils to plant odours and pheromones (Saïd *et al*. 2005), and factors affecting the responsiveness of beetles to their pheromones. Among the devices described in the literature (Baker & Lin 1984; Harari *et al.* 1994; Visser 1976), the four-arm olfactometer initially developed for small insects such as aphids, hymenopteran parasitoids or mites (Kaiser *et al.* 1989; Le Conte *et al.* 1989; Pettersson 1970; Vet *et al.* 1983) offered a design *a priori* favourable for studying synergistic responses to semiochemicals in a choice situation and has several advantages: it presents a dynamic system, with permanent reverse-choice possibilities and high statistical power (Kaiser *et al.* 1993; Vet *et al.* 1983). In

Correspondence to: Imen Saïd, e-mail: imene.said@univ-rennes1.fr

addition, it retains its properties when enlarged to study 1-2 cm long insects such as honeybees (Bakchine-Huber *et al.* 1992; Pham-Delegue *et al.* 1991, 1993) or West Indies sugarcane weevils (Ramirez-Lucas *et al.* 1996). Nevertheless, enlarging an olfactometer to a size adapted to stocky palm beetles generated many difficulties and required a new longer set up. In addition, to precisely control volatile delivery to insects, we used the volatile delivery system described by Bartelt and Zilkowski (1998). As the volatile delivery system and the four-arm olfactometer are two dynamic systems with opposite air flow controls (air respectively blown or drawn), the coupled system had to be adapted. Here, we investigated its physical functioning and biological relevance for the American palm weevil, *Rhynchophorus palmarum* and the dynast beetle, *Strategus aloeus*. The suitability of the olfactometer, without volatile delivery system, was evaluated for another dynast beetle, *Oryctes rhinoceros*.

Materials and methods

Insects

The palm weevil *R. palmarum* came from Colombia and French Guyana, the dynast scarabs *S. aloeus* and *O. rhinoceros* came from Colombia and Indonesia, respectively. The palm weevil is 40- to 45-mm long, rather flat, and the scarabs are 50- to 70-mm long and very stout, especially the major males. We tested field-collected insects that had been brought subsequently to France. Males and females were kept separately on sugarcane under tropical conditions (23-28 °C; R.H.: 75-90 %; L13:D11). Ten to 15 days conditioning to their new photoperiod were necessary to eliminate any physiological disturbance due to the time lag. One day before tests, insects were isolated in small boxes without food. Half an hour before tests, subjects were transferred to the bioassay room and allowed to acclimate for at least 30 min.

Physical set-up

Olfactometer

The four-arm olfactometer (Laucoin s.a., Thoiry, France) had the same shape as the one described by Pettersson (1970; in Vet 1983), but was enlarged ($55 \times 55 \times 5.5$ cm) (Fig. 1). Due to its size, we had to integrate specific elements to minimize the turbulence of airflows; plastic clamps (cl) were added to improve the air-tightness in the four corners of the olfactometer. Either stainless steel (5-mm thick porous sheet; Syntech, Hilversum, the Netherlands) or plastic (Mamison, Scamark, Issy-les-Moulineaux, France) grids (gd) were used to create laminar airflows at the inputs ($\varnothing = 20$ mm) and the output ($\varnothing = 60$ mm).

The olfactometer was made of opaque high density polyethylene (HDPE) with a polymethyl methacrylate (PMMA) transparent lid. It consisted in three parts: - the base with the air output, - the intermediate part, which delimited the walking chamber (3-cm high, 1-l volume) with the four air inputs, and - the lid (1-cm thick) with a 9-cm circular central opening to introduce insects. Two Neoprene® rings and four large thumb screws ensure the tightness between the three parts.

A diaphragm pump (Cole Parmer, U.S.A.) was used to draw air at constant flow rates. Four flow-meters (fm; Brooks, U.S.A.) controlled airflows into the olfactometer. We used the same constant flow rates in all four arms. Air was brought to the olfactometer through Tygon® 2275 plastic tubes (ty; ID = 10 mm) that had high chemical inertia and good flexibility.

Initially we reproduced Vet *et al.*'s (1983) protocol to check air-tightness of the device and turbulence of airflows in the central zone of the exposure chamber. The pump sucked white ammonium chloride smoke (HCl reacting with NH_4OH) onto a dark paper (Ecokraft; Maildor, France) stuck on the floor of the olfactometer chamber. However, as the ammonium chloride particles rapidly blocked the air inputs and stuck onto the lid, observation of the flows was impossible. Nevertheless, the acid vapours had a discolouring effect on the paper. Therefore, we used this acid property. Two opposite arms were connected to the acid flasks and ventilated for 5 min, and this induced clear discolouration. Five configurations (A: no grids; B, C: plastic grids either in the air inputs or output; D: plastic grids in both air inputs and output; and E: stainless steel grids in both air inputs and output), were tested for four different airflow rates (150, 250, 500 and 1000 ml/min per arm). Each configuration was evaluated four times for each flow rate.

The acidic prints on paper were recorded with a Tri CDD JVC KY camera equipped with a macro-photographic objective (Microcoputer-Nikkor 60 mm F: 2.8). The camera was connected to a microcomputer (Apple Macintosh Quadra 650) *via* a digitisation card (RGB Image Grabber 24). The images were processed either with Photoshop v. 4.0 (Adobe Systems Inc., U.S.A.) or Picture Publisher V. 6.0 (Micrografx Inc., U.S.A.) software.

Volatile delivery system

Our volatile delivery system (Fig. 1) was an adaptation of Bartelt and Zilkowski's (1998) model. Our system contained the same elements except that we used a capillary Teflon® tube (restrictor tube; ID = 0.5 mm, L = 5 cm) instead of a stainless steel needle to create the back pressure read on the manometer (ma). The odour source was a gently ventilated (9 ml/min) silanised glass vial (Sou: 250 ml; Fig 1) filled with 100 ml of a diluted aqueous solution of a volatile organic compound (VOC). Part of the VOC evaporated until equilibrium between head space concentration and aqueous concentration was reached (Henry's law; Betterton 1992). Mass delivery rate of the VOC was then constant. The percentage of the mass delivery rate (10-100 %) oriented to the mixture zone was regulated by a precision valve (V1); the remainder was evacuated through a low vacuum (Vac). The percentage of the mass delivery rate oriented to the mixture zone was correlated to the manometric difference indicated by the manometer (ma). Delivery rate of air flows from the mixture zone to the olfactometer could be regulated by eliminating part of the air flow through a vacuum (Vac) using an On/Off valve (V2). Several independent sources can be connected to the mixture zone (Fig. 1).

We followed Bartelt and Zilkowski's (1998) procedure to set up the volatile delivery system. This consisted in determining: first, the constant that governed the equilibrium of the pheromone source (Henry's constant; Bartelt 1997); second, the concentration delivered in the air at the device output and, third, the relationships between these values and the associated manometric differences obtained by adjusting valves V1 (Fig. 1).

Analytical chemistry

Solid phase microextration (SPME) was used to sample the VOCs in gaseous phase from the source. An SPME fibre was inserted into the tube carrying the airflow from the flask (Fig. 1: S1) and exposed to the VOC-laden air for 30 min. This duration was sufficient to obtain equilibration. The fibre coating was 100 µm PDMS (Supelco, Bellefonte, USA). The affinity constants of the fibre for the VOCs were determined according to Bartelt (1997). FID response factors of the chromatograph to the VOC were established from serial dilutions. Therefore, concentrations of VOC in the airflow coming from an odour source (ng/ml) were calculated directly from the GC peak area. These concentrations and the volumetric airflow rates (9 ml/min) gave the mass delivery rates of the VOC (ng/sec).

We used the male aggregation pheromone of the palm weevil *Rhynchophorus palmarum* to calibrate our volatile delivery system (see Bioassay section for chemical characteristics). SPME samples were analysed with a Varian Star 3400 Cx gas chromatograph equipped with a flame ionization detector (FID) and a WCOT

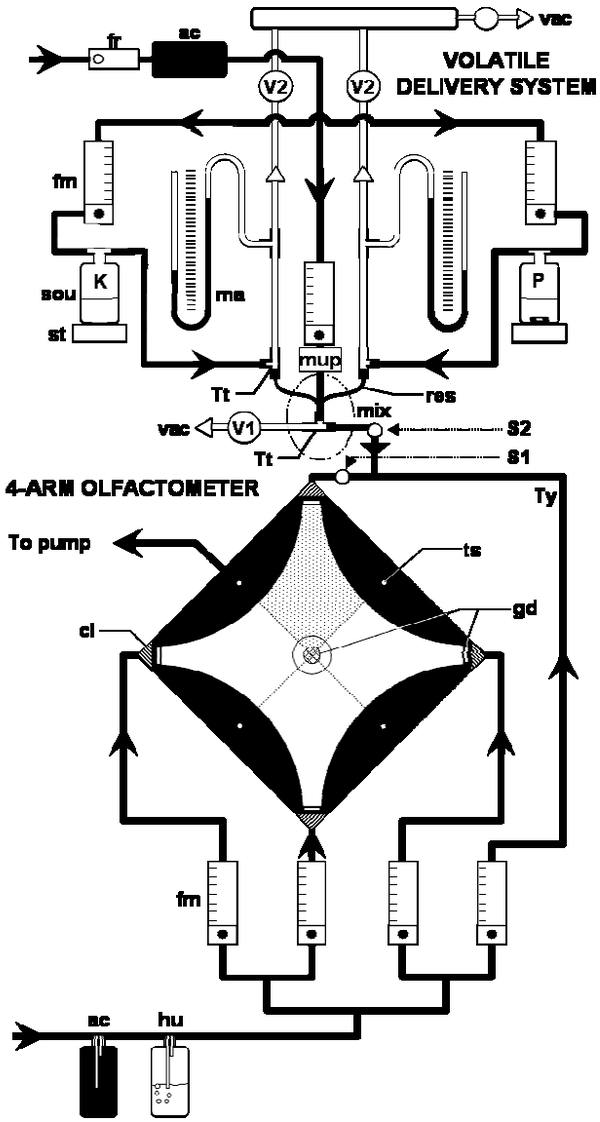

Fig. 1 Diagram of the large four-arm olfactometer coupled to the volatile delivery system set-up to deliver a binary mixture. **ac**: activated charcoal, **cl:** corner clamp, **fm**: flow-meter; **fr:** flow regulator; **gd:** stainless steel grid for straightening the air; **hu:** humidifier; **K**: kairomone; **ma:** manometer; **mix:** mixing zone; **mup:** makeup air; **P:** pheromone; **pump:** diaphragm pump; **res:** restrictor (capillary Teflon® tube); **S1, S2:** SPME sampling points to measure semiochemical concentrations in the air from the sources of the odour delivery system connected or not to the olfactometer; **sou:** volatile semiochemical source (diluted aqueous solution); **st:** magnetic stirrer; **ts:** large thumb screw, **Tt:** Teflon® tee (splitter); **V1, V2**: valves 1 and 2; **vac:** vacuum. **Ty:** Tygon® 2275 tubing

CPSil8-CB column (25 m × 0.32 mm (ID) × 0.4 µm d.f.). To calibrate the pheromone of *R. palmarum*, the column was operated from 50 °C (for 1 min) to 70 °C at 20 °C/min (plateau for 5 min), then to 250 °C (maintained finally for 3.5 min) at 8 °C/min. The injector and detector were heated at 230 and 260 °C, respectively. To calibrate the pheromone of *S. aloeus*, the column was operated from 15 °C (for 1 min) to 40 °C at 10 °C/min (plateau for 1 min), then to 80 °C at 5 °C/min (plateau for 1 min) and to 240 °C (maintained finally for 1.5 min) at 20°C/min. The injector and detector were heated as previously.

Olfactometer bioassays

First, the four-arm olfactometer was tested without coupling to the volatile delivery system. We studied the response of *Rhynchophorus palmarum* males and females to extracts of natural volatiles (males feeding on sugarcane). We established the dose-response of *O. rhinoceros* to synthetic aggregation pheromone. The stimulus was deposited on a 15 × 15 filter paper placed in a 100 ml glass flask connected to an olfactometer input. The volatile delivery system could not be used for *O. rhinoceros* pheromone because it is not very soluble in water.

Second, responses of *R. palmarum* and *S. aloeus* to their synthetic pheromones were tested by coupling the four-arm olfactometer to the volatile delivery system.

Odours tested

The odour stimuli were prepared as follows:

R. palmarum - Natural volatile extracts containing *R. palmarum* pheromone and sugarcane volatiles. We sampled the odours emitted by 10 males fed on sugarcane for three days. Insects and food were placed in a 1-l glass flasks connected to a cartridge filled with

an adsorbent (Supelpak-2, Supelco). An empty flask was prepared for control. Moistened and charcoal-purified air was drawn through the device at 100 ml/min. The adsorbent-trapped odours were eluted by 4 ml dichloromethane and concentrated under a nitrogen flow. GC analyses showed that the final pheromone concentration was 18 ng/µl. We used 20 µl of this extract, deposited on filter papers previously treated with 10 µl mineral oil (Sigma Chemical Co., U.S.A.) and renewed for each test. A solvent control was delivered through another randomly chosen arm.

The synthetic pheromone (Rochat *et al.* 1991), 2-methyl-(5*E*)-hepten-4-ol (purity > 98 % by gas chromatography [GC]; E.G.N.O.-Chimie, Tancarville, France), was diluted in ultra-pure water. At a pheromone concentration of 5 µg/ml with valve V1 open to give 50 % of the total delivery rate, a mass delivery rate of 1.1 ± 0.1 ng/s passed into the olfactometer. At a pheromone concentration of 1 µg/ml with valve V1 open to give 10 % of the total delivery rate, a mass delivery rate of 30 pg/s passed into the olfactometer.

S. aloeus - The synthetic pheromone (Rochat *et al.* 2000b) is a mixture (98:1:1 (v/v)) of 2-butanone (> 99.5 % GC; Janssen, Belgium), 3-pentanone (> 98 % GC; Aldrich, Gillingham, U.K.) and *sec*-butyl acetate (> 99 % GC; Aldrich, Milwaukee, U.S.A.). An aqueous solution of the pheromone was prepared at 50 ng/ml. The valve V1 was fully open and allowed 100 % of the total delivery rate to pass into the olfactometer, yielding 25 pg/s of the pheromone.

O. rhinoceros - The synthetic pheromone (Hallett *et al.* 1995) is ethyl 4-methyloctanoate (> 98 % GC; E.G.N.O.-Chimie). Six pheromone solutions in high grade hexane (10^{-1} to 10^{5} ng/µl; decadic steps) were prepared. One µl of pheromone solution was deposited on a 15 × 15 mm filter paper previously treated with 10 µl mineral oil (Sigma Chemical Co., U.S.A.) for each test. A solvent control was delivered through another randomly chosen arm.

Bioassay procedure

We used configuration E (Fig. 2) with a flow rate of 500 ml/min in each arm. To facilitate movements of insects, a plastic lattice (4 mm^2 mesh, 1 mm thick) was stuck onto the floor of the exposure chamber. Bioassays were conducted under the red light when the activity of these insects peaked. *R. palmarum* and *O. rhinoceros* were tested within the first three hours of the scotophase, at 25 ± 1 °C. *S. aloeus* were tested between the second and the sixth hour of the scotophase, at 28° ± 1 °C. Insects were placed individually in the centre of the olfactometer. Tests lasted 10 min. Movements of insects in the different fields were recorded by an adapted data-processing software (EVEN v. 1.0; INRA, France). Each of the four fields was considered a separate zone, when recording beetle positions and responses to test volatiles. A fifth zone, CZ, in the exposure chamber corresponded to a 9 cm circular central zone where the airflows from the four arms mixed.

We followed the insects visually and recorded their entries into the different fields with the keyboard. EVEN software (INRA, France) calculated time spent in each field. A test began once the insect started to move. We considered that an insect entered a given field when its entire thorax crossed the zone boundary. A test was not retained when - an insect remained motionless in the central zone for more than 5 min or in one field for more than 9 min, - it fell on its back more than six times, or - time spent in the central zone was more than 10 % of the test duration. Seventeen to 20 *R. palmarum* and *O. rhinoceros* (ca. 1:1 male/female ratio) and 20 to 23 *S. aloeus* of each sex were tested for each dose.

Approximately 10-15 bioassay tests could be conducted with the olfactometer in one day. Assignment of treatments to the olfactometer arms was the same throughout a test day but was re-randomised for the next test day.

Response criteria and statistical analyses

Two criteria quantified behaviour: 1- time spent in each field; 2- number of insects that chose the odorised field at the beginning of the test (first choice). One-way ANOVAs compared mean times spent in each of the four fields [$(x+0.5)^{1/2}$ transformed data; insects as blocks] followed by Newman-Keuls' multiple comparison tests (Dagnelie 1975). Binomial tests with an equivalent probability to visit each field ($P = 0.25$ for the odorised field *versus* $P = 0.75$ for the other fields; Siegel 1956) evaluated significance of first choices.

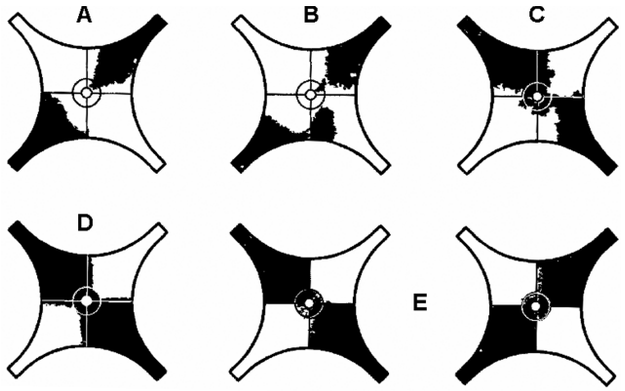

Fig. 2 Digitized acidic prints on sensitive paper evidencing air flow paths and limits (500 ml/min) in relation to configuration (no grids [A]; plastic [B-D] or stainless steel [E] grids). Doted lines: theoretical limits of the odour fields of the four arms and the central zone where odours mixed in every configuration. Configuration E was chosen for bioassays

Results and discussion

Vet *et al*.'s (1983) protocol, using smoke to visualize airflows in a four-arm olfactometer was incompatible with the use of our thin mesh grids. Therefore, visualizing airflows by discolouring sensitive papers with acid vapours proved to be an interesting method to determine airflow limits and paths. Airflows were visualized for four airflow rates and five configurations of the set-up. In the first configurations (no grids, or only either in the air inputs or in the output; Fig. 2A-C) airflows were turbulent and extended beyond the theoretical limits of each field into the exposure chamber. Stainless steel grids yielded symmetrical prints showing that the atmospheres of the different fields did not mix (Fig. 2E). Therefore we chose configuration E for bioassays. Among the four different airflow rates tested, the 500 ml/min airflow rate gave the best digitized acidic prints (Fig. 2). However, the 150 ml/min flow gave a discolouration gradient decreasing from the airflow input to the central air output. On the other hand, the 1000 ml/min flow appeared too strong. Therefore, we chose the 500 ml/min flow for bioassays.

A circular central zone (CZ: ∅ = 9 cm) in the exposure chamber surrounding the central air output was discoloured independently of the field tested. The airflows from the four fields mixed in this central zone. This was not an inconvenience and, in fact, allowed the large beetles to perceive all the odours at the beginning of a test. Therefore, we adapted our recording procedure by adding a fifth zone: CZ.

The aqueous solution of *R. palmarum* pheromone reached Henry's equilibrium in about 2 h that was maintained for 4-5 h under our experimental conditions. Relationships

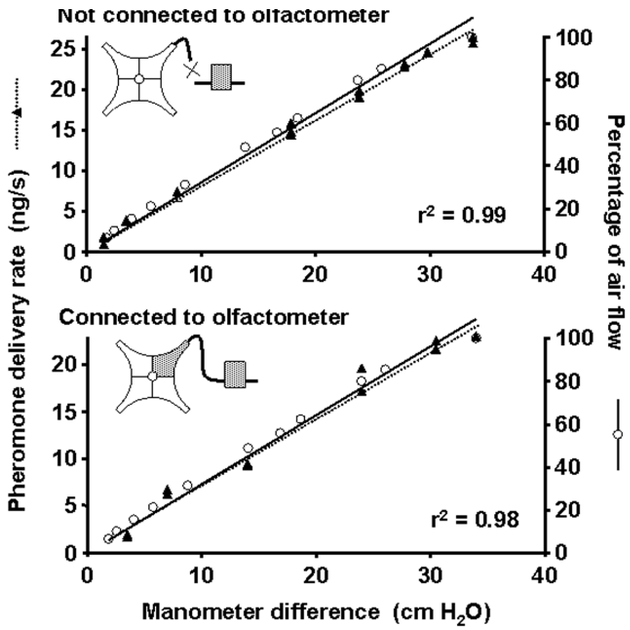

Fig. 3 Relationships between manometer differences (cm H_2O) *versus* pheromone (rhynchophorol) delivery rates (black triangles) and the air flow calibration curve (open circles), in two situations: volatile delivery system connected (top) or not (bottom) to the four-arm olfactometer. The aqueous pheromone solution (50 µg/ml) was ventilated by 9 ml/min charcoal-purified air at 25 °C

between airflow rates through the restrictor tube and pressure readings on the manometer were linear, whether the volatile delivery system was connected to the olfactometer or not ($r^2 = 0.99$) (Fig. 3). Quantities of pheromone delivered by the volatile delivery system, connected or not to the olfactometer, were proportional to manometric differences (Fig. 3; linear adjustment r^2 of 0.99 and 0.98, respectively). Coupling the volatile delivery system to the olfactometer did not influence VOC delivery rates for the same manometric difference.

Coupling the four-arm olfactometer to the volatile delivery system did not impair the physical functioning of either of the two devices. One advantage of the volatile delivery system was the absence of organic solvents, thus providing true blank controls and thereby eliminating risks that insects responded to the solvent. The only delicate factor was the intrinsic sensitivity of the volatile delivery system to temperature variations, because it was based on thermodynamics equilibrium (Betterton 1992). Nevertheless, in the thermostatic experimental room, small temperature variations (1 °C) had negligible effects on the pheromone mass flow actually delivered (2 to 5 %).

All three species responded significantly to their synthetic pheromones (Fig. 4-5). In most test series data for only one or two, out of a dozen insects, had to be discarded (no reaction after 5 min). Insects generally spent less than 1 min in the central zone. They entered the odorized field quickly when presented most of the tested odours. When insects moved out of the odorized field into an adjacent field, they tended to stop for a short time, to pick up their antennae, and then to return to the odorized field. Although these beetles are good flyers and approach their host plants by flight, they walked most of the time in the olfactometer. *O. rhinoceros* made a few flying attempts and when they occurred tests aborted rarely and only if beetles fell on their backs more than six times.

When presented extracts of natural volatiles in the four-arm olfactometer, *R. palmarum* spent only a short time in the central zone (< 1 min) and chose rapidly one of the odour fields. Males (n = 20) and females (n = 14) chose the field odorized by the natural extract, respectively in 75 and 78 % of the tests ($P < 0.001$; binomial test). As soon as insects crossed this odourized field towards an adjacent one, they stopped for a moment, straightened their antennae and then went back to the odourized field. Insects spent significantly more time in the odourized field than in the other fields (females: $F(3,39) = 14.02$; $P < 0.001$; males: $F(3,57) = 15.92$; $P < 0.001$) (Fig. 4).

Oryctes rhinoceros spent significantly more time in the pheromone field than in the control field (blank and solvent) when presented 10^3 to 10^5 ng doses on filter paper (Fig. 5). When presented 10 ng doses, time spent in the pheromone field did not differ significantly from time spent in the solvent control field (27 ± 8 % test duration), but was significantly greater than time spent in the blank field (14 ± 3 and 17 ± 6 % test duration). The preference for the pheromone field reached a plateau at the three highest doses (10^3, 10^4 and 10^5 ng) (between 42 ± 4 % and 45 ± 5 % test duration). The first choices (50, 70, 70, 72 and 68 % of the tests) for the pheromone field were significant from 1 to 10^5 ng ($P < 0.0$; binomial test).

R. palmarum responded similarly to a constant concentration of the pheromone delivered by the volatile delivery system. Only two tests had to be discarded as those insects had not reacted after 5 min. Insects chose significantly the pheromone field at the beginning of 62 and 75 % of the tests when stimulated by 30 pg/s and 1.1 ng/s respectively (n = 20; $P < 0.001$; binomial test). They remained in the pheromone field (40 ± 4 % and 64 ± 5 %) significantly longer than in the control field when stimulated by 30 pg/s ($F(3,57) = 25.19$; $P < 0.001$) and by 1.1 ng/s ($F(3,57) = 7.95$, $P < 0.05$) (Fig. 4).

Male and female *S. aloeus* responded differently to the synthetic aggregation pheromone dispensed by the volatile delivery system. Females spent significantly more time in the pheromone field (46 ± 8 % of the test duration) than in the control fields ($F(3, 57) = 8.27$, $P < 0.05$) (Fig. 4). The pheromone field was chosen first by 60 % of the females ($P < 0.01$, binomial tests). Males spent as long in the pheromone field as in the control fields. At the beginning of the tests, males avoided the pheromone field and 52 % of them chose the opposite field ($P < 0.005$, binomial tests).

Behaviour of palm beetles was reliable and replicable in the four-arm olfactometer. The fact that a high proportion of the subjects chose the odourized field first, where, in addition, they spent the most time, showed that these insects could perceive and discriminate the odour and the limits of each field and confirmed that the atmospheres of the fields did not mix.

Insects responded to much lower doses of pheromones and significant responses were obtained with fewer individuals in the four-arm olfactometer, whether it was coupled or not to the volatile delivery system, than in the pitfall or

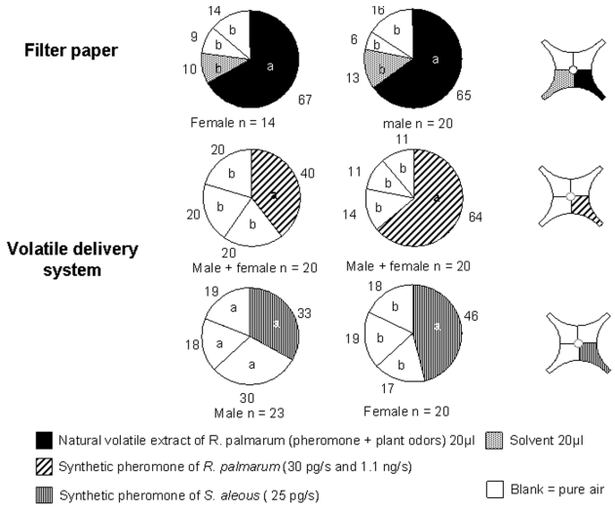

Fig. 4 Responses of *Rhynchophorus palmarum* and *Strategus aloeus* in the four-arm olfactometer to natural extracts and synthetic aggregation pheromones after 10 min. The natural extract of volatiles emitted by *R. palmarum* on sugarcane (natural pheromone and plant odours) was deposited on filter paper (20 µl). The synthetic aggregation pheromones of *R. palmarum* (1.1 ng/s and 0.03 ng/s) and of S. aleous (25 pg/s) were dispensed by the volatile delivery system. Mean times spent in each of the four fields with the same letters do not differ significantly (Newman-Keuls' tests; $P < 0.05$)

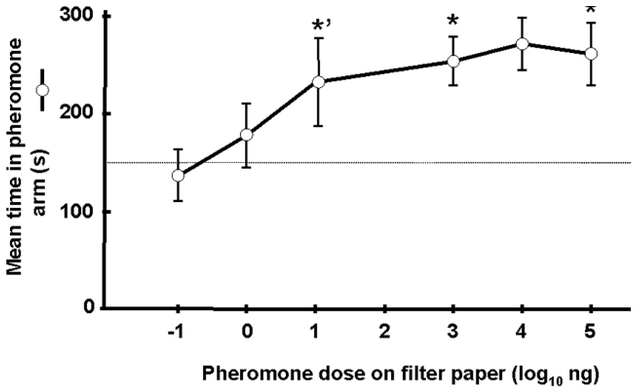

Fig. 5 Responses of *Oryctes rhinoceros* (17-20 males and females) in the four-arm olfactometer to various doses of synthetic pheromone (ethyl 4-methyl octanoate; hexane solution) emitted from filter papers after 10 min. Mean time spent in the pheromone field (significant differences are indicated by: * different from all control fields, and *' different only from blank control arms, Newman-Keuls' tests; $P < 0.05$). Bars represent standard errors of the means

Y-tube olfactometers. *R. palmarum* appeared less stressed in this olfactometer than in a pitfall olfactometer or in a modified wind tunnel (Rochat *et al.* 1991, 2000a). In a pitfall olfactometer, 10 ng of aggregation pheromone on a filter paper and 63 *R. palmarum*, each tested for 30 min, were necessary to obtain a significant result. Here, a significant result was recorded with only 20 weevils tested for 10 min and using 30 pg/s or 1.1 ng/s pheromone delivery rates. In a pitfall olfactometer, *O. rhinoceros*, reacted only to pheromone doses equal or greater than 1 µg, and 80 insects were necessary to obtain a significant result (Rochat, unpublished data), whereas results were significant in the four-arm olfactometer for 10 ng/s and 17 insects. Stimulation by constant controlled concentrations yields a more precise measure of the response threshold of insects to chemical signals. Hallett *et al.* (1995) reported positive responses of this species, but they used as much as 612 ng pheromone (600 ng ethyl 4-methyloctanoate + 12 ng ethyl 4-methylheptanoate) and 68 individuals in a Y-tube olfactometer; they did not report the number of non-responding beetles. *S. aloeus* females reacted significantly to about 1 000 times less pheromone (15 ng/10-min test) when 20 µg was deposited on filter paper (Rochat *et al.* 2000b). Reactions of males at the lowest doses showed that some individuals clearly avoided the pheromone.

The literature gives few examples of laboratory studies of the responses of large flying Coleoptera to semiochemicals. In most cases, these studies either used large devices (wind tunnels or Y-shaped tubes) or were conducted in bioassay rooms: *e.g.* cerambycids, responding to either kairomones (*Monochamus alternatus*, Hope; Sakai & Yamasaki 1990) or pheromones (*Xylotrechus pyrrhoderus,* Bates; Iwabuchi *et al.* 1986). Despite extensive field experiments, responses of palm beetles to semiochemicals have rarely been investigated in the laboratory (Gunawardena *et al.* 1998; Hallett *et al.*1995; Jaffé *et al.* 1993; Rochat *et al.*1991). Coupling gas-chromatography with electroantennography allows the identification of semiochemicals potentially active for palm beetles (*e.g.* Gries *et al.* 1994a, b; Gunawardena *et al.* 1998). This technique can be used for screening, but it cannot give any indications about the behavioural impact of the compounds. Laboratory bioassays, other than ours, implied generally Y-shaped olfactometers, operated with high doses of semiochemicals (> 10 µg; *op. **cit.*** **references**). Various sizes of the four-arm olfactometer have been used successfully to study hymenopteran parasitoids, the honeybee *Apis mellifera*, and the sugarcane weevil *Metamasius hemipterus* (Pham-Delègue *et al.* 1991; Ramirez-Lucas *et al.* 1996; Vet *et al.* 1983), but no olfactometer large enough to house palm weevils had previously been tested.

To conclude, coupling the volatile delivery system to the four-arm olfactometer provides an efficient tool to deliver low doses of very volatile compounds such as the pheromone of *S. aloeus* and plant odours, and to eliminate the presence of solvents that, in some cases, can have an attractive or repellent effect. As shown by Bartelt and Zilkowski (1998), the volatile delivery system controls concentrations of natural stimuli and synthetic stimuli with the same efficiency. This property is interesting as it allows investigation of the effect of natural compounds at realistic doses. Recently, a six-arm olfactometer, developed by Turlings *et al.* (2004), enabled observation of insect attraction to simultaneously trapped natural odours. The advantage of our system is the possibility to modulate odour doses using the same source. The four-arm olfactometer coupled to the volatile delivery system is suitable for different large beetles and should enable investigation of their behaviour such as responses to several semiochemicals in multi-choice

configurations. Saïd *et al.* (2005), evidenced a clear synergistic response of male and female *R. palmarum* to mixture of pheromone and plant odour (acetoin), using the four-arm olfactometer. Detailed dose-response relationships and synergy evaluation to pheromone and plant odour in *R. palmarum*, using the volatile delivery system, are reported in Saïd & Rochat (submitted).

Acknowledgements

We thank Rosa Aldana for providing *R. palmarum*, Jan Van der Pers for making the stainless steel grids for our olfactometer and Ann Porter Cloarec for revising the English text.

References

Abraham VA, Faleiro JR, Al-Shuaibi MA, Prem Kumar T (2000) A strategy to manage red palm weevil *Rhynchophorus ferrugineus* Oliv. on date palm *Phoenix dactylifera* L. Its successful implementation in Al-Hassa, Kingdom of Saudi Arabia. Pestology 24: 23–30

Bakchine-Huber E, Marion-Poll F, Pham-Delegue MH, Masson C (1992) Realtime detection and analysis of the exploratory behavior of small animals. *Naturwissenschaften* 79: 39–42

Baker TC, Carde RT (1984) Techniques for behavioral bioassays. Pp 45–73 in Hummel HE, Miller TA (eds) Techniques in Pheromone Research

Baker TC, Lin CE (1984) Wind tunnels in pheromone research. Pp 75–110 in Hummel HE,. Miller TA (eds) Techniques in Pheromone Research

Bartelt RJ (1997) Calibration of a commercial solid-phase microextraction device for measuring headspace concentrations of organic volatiles. Anal Chem 69: 364–372

Bartelt RJ, Zilkowski BW (1998) A versatile and quantitative volatile-delivery system for laboratory bioassays. J Chem Ecol 48: 535–558

Bedford GO (1980) Biology, ecology, and control of palm Rhinoceros beetles. Ann Rev Entomol 25: 309–339

Betterton EA (1992) Henry's law constants of soluble and moderately soluble organic gases: Effects on aqueous phase chemistry. Pp 1–47 in NRIAGU JO (eds) Gaseous pollutants: characterisation and cycling

Chung GF (1997) The bioefficacy of the aggregation pheromone in mass trapping of rhinoceros beetles (*Oryctes rhinoceros* L.) in Malaysia. The Planter (Kuala Lumpur) 73: 119–127

Dagnelie P (1975) Théorie et méthodes statistiques, applications agronomiques, Vol. 2. Les presses agronomiques de Gembloux, Belgium 463 pp

Giblin-Davis RM, Oehlschlager AC, Perez AL, Gries G, Gries R, Weissling TJ, Chinchilla CM, Pena JE, Hallett RH, Pierce JR HD, Gonzalez LM (1996) Chemical and behavioral ecology of palm weevils (Curculionidae: Rhynchophorinae). Fla Entomol 79: 153–167

Gries G, Gries R, Perez AL, Oehlschlager AC, Gonzales LM, Pierce JR HD, Zebeyou M, Kouame B (1994a) Aggregation pheromone of the African rhinoceros beetle, *Oryctes monoceros* (Olivier) (Coleoptera: Scarabaeidae). Z Naturforsch 49: 363–366

Gries G, Gries R, Perez AL, Gonzales LM, Pierce JR HD, Oehlschlager AC, Rhainds M, Zebeyou M, Kouame B (1994b) Ethyl propionate: synergistic kairomone for African palm weevil, *Rhynchophorus phoenicis* L. (Coleoptera: Curculionidae). J Chem Ecol 20: 889–897

Gunawardena NE, Kern F, Janssen E, Meegoda C, Schäfer D, Vostrowsky O, Bestmann HJ (1998) Host attractants for red weevil, *Rhynchophorus ferrugineus*: identification, electrophysiological activity, and laboratory bioassay. J Chem Ecol 24: 425–437

Hallett RH, Perez AL, Gries G, Gries R, Pierce JR HD, Yue J, Oehlschlager AC, Gonzalez LM, Borden JH (1995) Aggregation pheromone of coconut rhinoceros beetle, *Oryctes rhinoceros* (L.) (Coleoptera: Scarabaeidae). J Chem Ecol 21: 1549–1569

Harari AR, Ben Yakir D, Rosen D (1994) Mechanism of aggregation behavior in *Maladera matrida* ARGMAN (Coleoptera: Scarabaeidae). J Chem Ecol 20: 361–371

Hardie J, Minks AK (eds.) (1999) Pheromones of Non-Lepidopteran Insects Associated with Agricultural Plants. CABI Publishing, Wallingford, UK, 466 pp

Iwabuchi K, Takahashi J, Nakagawa Y, Sakai T (1986) Behavioral responses of female grape borer *Xylotrechus pyrrhoderus* Bates (Coleoptera: Cerambycidae) to synthetic male sex pheromone components. App Entomol Zool 21: 21–27

Jaffe K, Sanchez P, Cerda H, Hernandez JV, Jaffe R, Urdaneta N, Guerra G, Martinez R, Miras B (1993) Chemical ecology of the palm weevil *Rhynchophorus palmarum* (L.) (Coleoptera: Curculionidae): attraction to host plants and a male produced aggregation pheromone. J Chem Ecol 19: 1703–1720

Kaiser L, Pham-Delegue M-H, Bakchine H, Masson C (1989) Olfactory responses of *Trichogramma maidis* Pint. & Voeg: effects of chemical cues and behavioral plasticity. J Insect Behav 2: 701–712

Le Conte Y, Arnold G, Trouiller J, Masson C, Chappe B, Ourisson G (1989) Attraction of the parasitic mite *Varroa* to the drone larvae of honey bees by simple aliphatic esters. Science 245: 638–639

Oehlschlager AC, Chinchilla C, Castillo G, Gonzalez L (2002) Control of red ring disease by mass trapping of *Rhynchophorus palmarum* (Coleoptera: Curculionidae). Fla Entomol 85: 507–513

Oehlschlager AC, McDonald RS, Chinchilla CM, Patschke SN (1995) Influence of a pheromone-based mass-trapping system on the distribution of *Rhynchophorus palmarum* (Coleoptera: Curculionidae) in oil palm. Environ Entomol 24: 1005–1012

Oehlschlager AC, Pierce JR HD, Morgan B, Wimalaratne PDC, Slessor KN, King GGS (1992) Chirality and field activity of Rhynchophorol, the aggregation pheromone of the American palm weevil. Naturwissenschaften 79: 134–135

Petterson J (1970) An aphid sex attractant. I. Biological studies. Entomologica Scand 1: 63–73

Pham-Delegue MH, Trouiller J, Bakchine E, Roger B, Masson C (1991) Age dependency of worker bee response to queen pheromone in a four-armed olfactometer. Insect Sociol 38: 283–292

Pham-Delegue MH, Trouiller J, Caillaud J, Roger B, Masson C (1993) Effect of queen pheromone on worker bees of different ages: behavioural and electrophysiological responses. Apidol 24: 267–281

Ramirez-Lucas P, Malosse C, Ducrot P-H, Lettere M, Zagatti P (1996) Chemical identification, electrophysiological and behavioral activities of the pheromone of *Metamasius hemipterus* (Coleoptera: Curculionidae). Bioor Med chem 4: 323–330

Rochat D, Malosse C, Lettere M, Ducrot P-H, Zagatti P, Renou M, Descoins C (1991) Male-produced aggregation pheromone of the american palm weevil, *Rhynchophorus palmarum* (L.) (Coleoptera: Curculionidae), collection, identification, electrophysiological activity and laboratory bioassay. J Chem Ecol 17: 1221–1230

Rochat D, Nagnan-Le Meillour P, Esteban-Duran JR, Malosse C, Perthuis B, Morin J-P, Descoins C (2000a) Identification of pheromone synergists in American palm weevil, *Rhynchophorus palmarum*, and attraction of related *Dynamis borassi* (Coleoptera, Curculionidae). J Chem Ecol 26: 155–187

Rochat D, Ramirez-Lucas P, Malosse C, Aldana R, Kakul T, Morin J-P (2000b) Role of solid phase micro-extraction in the identification of highly volatile pheromones of two rhinoceros beetle *Scapanes australis* and *Strategus aloeus* (Coleoptera, Scarabaeidae, Dynastinae). J Chromatogr A 885: 433–444

Rochat D, Morin JP, Kakul T, Beaudoin-Ollivier L, Prior R, Renou M, Malosse I, Stathers T, Embupa S, Laup S (2002) Activity

of male pheromone of the Melanesian rhinoceros beetle *Scapanes australis*. J Chem Ecol 28: 479–500
Rochat D, Mohammadpoor K, Malosse C, Avand-Faghih A, Lettere M, Beauhaire J, Morin J-P, Pezier A, Renou M, Abdollahi GA (2004) Male aggregation pheromone of date palm fruit stalk borer *Oryctes elegans*. J Chem Ecol 30: 387–407
Sakai M, Yamasaki T (1990) (+)-juniperol and (+)-pimaral: attractants for the cerambycid beetle, *Monochamus alternatus* Hope. J Chem Ecol 16: 3383–3392
Saïd I, Renou M, Morin J-P, Ferreira JMS, Rochat D (2005) Interactions between acetoin, a plant volatile and pheromone in *Rhynchophorus palmarum*: behavioral and olfactory neuron responses. J Chem Ecol 31: 1789–1805
Siegel S (1956) Non parametric statistics for the behavioral sciences. McGraw Hill Book Company, New York 312 pp
Turlings TCJ, Davison AC, Tamo C (2004) A six-arm olfactometer permitting simultaneous observation of insect attraction and odour trapping. Physiol Entomol 29: 45–55
Vet LEM, Van lenteren JC, Heymans M, Meelis E (1983) An air flow olfactometer for measuring olfactory responses of hymenopterous parasitoides and other small insects. Physiol Entomol 8: 97–106
Visser, JH (1976) The design of a low-speed wind tunnel as an instrument for the study of olfactory orientation in the Colorado beetle (*Leptinotarsa decemlineata*). Entomol Exp Appl 20: 275–288

Received 17 May 2005; accepted 20 August 2005.
Published Online First 25 October 2005.